BRCA1's Phosphorylation Dependent Interaction with Abraxas, BACH1, and CtIP: Elucidation of the BRCA1 Mediated DNA Damage Response

Honors Research Thesis

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Abstract

Women carrying germline mutations of the BRCA1 gene show an increased risk of breast and ovarian cancer, implying that the BRCA1 protein acts as a tumor suppressor in mammary and ovarian epithelial cells. The rationale of this thesis is to elucidate how BRCA1 suppresses tumor development..

To investigate BRCA1's tumor suppression activity, our lab has recently developed a mouse model of the basal-like breast tumors that arise in women who carry BRCA1 mutations. Furthermore, this model was used successfully to define specific functions of BRCA1 that are and are not required for tumor suppression. Our lab has shown that deletion of Brca1 results in embryonic lethality, implying that the Brca1 protein is essential in embryonic development. Inactivation of Brca1 in mammary epithelial cells resulted in development of mammary tumors similar to the basal-like tumors observed in patients carrying BRCA1 mutations. These studies indicate that BRCA1 is essential for tumor suppression.

Many tumor-associated BRCA1 alleles have frameshift or nonsense mutations that delete one or both of the BRCT motifs (Fig. 1), suggesting that these motifs may operate either directly or through interaction with other proteins to suppress tumor development[1]. Our lab has shown that these BRCT repeats are essential for tumor suppression in part by playing a key role in homology directed DNA repair (HDR) of double-stranded breaks (DSBs), and repair of DNA interstrand cross-linking (ICL) damage. Specifically, the phosphoserine interaction of BRCA1 to other partners is essential in mediating error-proof DNA repair[1]. In addition, mice carrying Brca1 BRCT mutations are susceptible to spontaneous tumor development [1; 2]. These results indicate that the BRCT motifs in BRCA1 facilitate DNA repair and tumor suppression either directly and/or via interactions with other DNA repair proteins. In particular, we are interested in the

interaction with three phosphorylated isoforms of repair proteins: Abraxas, BACH1, and CtIP. The goal of this thesis is to elucidate the BRCT interacting partners of BRCA1 and their particular functions in maintaining genomic stability and facilitating DNA repair.

To accomplish this, our lab will cross mice to generate primary embryonic fibroblasts (pMEFs) containing mutations that ablate the phosphoserine interaction capability of the three phosphorylated isoforms of repair proteins,individually or in combination with the intact Brca1 protein. Further, a combination of mutations in the three repair proteins will be generated in cells with a deleted 53BP1 gene to study their specific role in ICL repair or HDR. Cells expressing these mutant alleles will be tested for sensitivity to DNA damaging agents, proliferation in culture, and HDR.

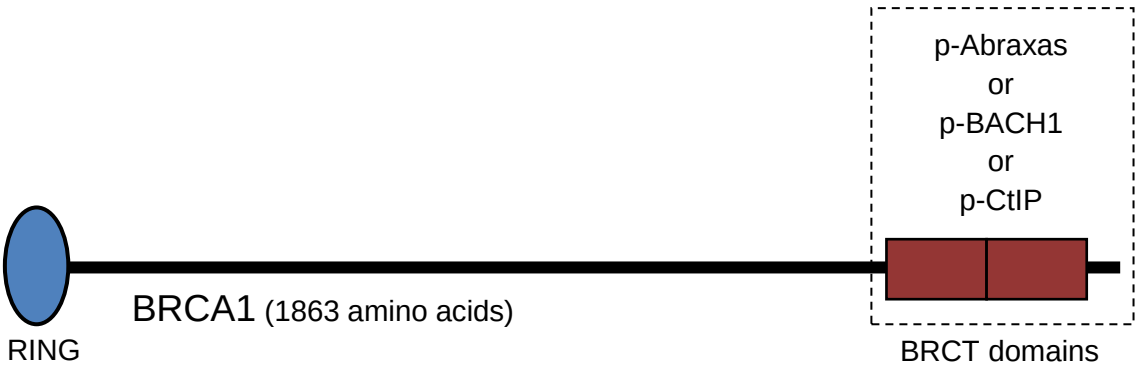


Figure 1. BRCA1 and it C-terminal BRCT repeats.

Introduction

Eukaryotic cells have evolved multiple strategies to counter a variety of DNA lesions, including DNA strand breaks[3]. The importance of DNA repair is vital, as deficiencies in a number of DNA repair pathways are linked to human disease and cancer[3]. In mammalian cells, HDR and nonhomologous end joining (NHEJ) are two major pathways of DSB repair[4]. HDR occurs during late S phase and early G2 phase of the cell cycle and leads to error-proof repair of DNA damage using the intact sister chromatid as a template[3]. During HDR, strand invasion of the damaged, single-stranded DNA into its double-stranded sister chromatid is initiated by localization and binding of RAD51 to sites of DSBs[3]. ssDNA-binding replication protein A (RPA) also localizes at sites of DSBs and may play a key role in promoting end resection to generate ssDNA[3]. HDR is a crucial pathway in repairing lesions that threaten genome stability, including DSBs and ICLs.

When the HDR pathway is inactivated, DNA repair is directed along more error-prone pathways, such as NHEJ where broken DNA ends are directly re-ligated[4]. Because NHEJ is not guided by a homologous template, cells accumulate chromosomal aberrations, deletions, translocations, and nucleotide substitutions[3]. Thus, error-prone pathways contribute to genome instability and are implicated in tumorigenesis[3].

Multiple studies have implicated the BRCA1 protein as a major facilitator of HDR and therefore, a tumor suppressor. BRCA1 lesions that are associated with cases of familial breast cancer are usually frameshift or nonsense mutations that result in a truncated polypeptide where one or both BRCT motifs are deleted[1]. Furthermore, some breast cancer cases are linked to a single amino acid substitution that ablates the phosphorylation-dependent interaction of the BRCT domain and its phospho-ligands[1]. These corresponding BRCA1 mutations were

introduced into mice to investigate BRCA1's role in tumor suppression and multiple pathways of DNA repair. As mentioned earlier, $Brca1^{-/-}$ mutations in mammary epithelial cells result in the development of mammary tumors. Previously, our lab generated three mouse models with mutations that deleted or disrupted the coding potential of the BRCT motifs or specifically disrupted the phospho-serine interaction of the BRCT domain with its cognate phospho-ligands: $Brca1^{tr/tr}$ mice express a truncated Brca1 polypeptide lacking the carboxy-terminal BRCT repeats, while the $Brca1^{M1717R/M1717R}$ and $Brca1^{S1598F/S1598F}$ mutations are a single amino acid substitution that ablate the phospho-dependent interaction of Brca1 and its partners. Mice homozygous for these Brca1 mutations experienced accelerated tumor development compared to wild type mice. MEFs with homozygous Brca1 mutations accumulated spontaneous chromosomal aberrations and were highly sensitive to DNA damaging agents including Poly ADP Ribose Polymerase inhibitor (PARPi), which inhibits single-stranded break repair, and mitomycin C (MMC) and cisplatin, which introduce ICL damage to DNA (Figure 2a, 2b)[1; 5]. In addition, these mutant MEFs were also deficient in HDR (Figure 3) and experienced senescence early on compared to wild type cells (Figure 4). Thus, the DNA repair and tumor suppression functions of BRCA1 are dependent on its ability to interact with one or more of its BRCT phospho-ligands.

Previous studies indicate that three isoforms of repair proteins, Abraxas, BACH1, and CtIP, when phosphorylated, preferentially bind to the BRCA1 BRCT domains *in vivo*[3]. Because BRCA1 binding to these three repair proteins is mutually exclusively, it potentially forms three distinct protein complexes that seem to influence the DNA damage response[3]. While it is unclear how each of the three protein complexes participate in DSB repair in their respective pathways, all three phospho-ligands seem to have independent, different contributions to DSB repair.

CtIP plays a central role in DNA resection by promoting recruitment of RPA to sites of DSBs[6]. In addition, studies on yeast show that CtIP is a target for cyclin-dependent kinase 1 (CDK1), a regulatory protein of DNA end resection during the cell cycle[6]. CDK phosphorylation of CtIP allows binding of the BRCT domains of BRCA1 and subsequently, 5' to 3' end resection during HDR[6].

BACH1 may regulate HDR by acting as a helicase in unwinding DNA at sites of DNA damage[3]. Deletion of BACH1 in human cells results in hypersensitivity and chromosomal instability after treatment with ICL-inducing agents[3]. After phosphorylation, BACH1 binds to BRCA1 BRCT repeats and seems to have helicase substrate affinity for secondary structures, including ICL damage[3]. BACH1, BRCA1, and a topoisomerase are loaded onto replication origins and facilitate DNA replication, timely S-phase progression, and loading of of RPA onto chromatin[3]. BACH1 has also been identified as a deficient protein in Fanconi anaemia (FancJ)[3].

The BRCA1 interaction with Abraxas forms the BRCA1-A complex involved in G2-M checkpoint control[3]. Abraxas along with other proteins form a complex that localizes on sites of DNA damage after phosphorylation and interaction with BRCA1[3]. This checkpoint control ensures that entry into mitosis is temporarily arrested to avoid aberrant chromosome segregation[3]. Abraxas seems to play a key role in localizing BRCA1 on sites of DNA damage through a phosphorylated-dependent interaction.

The phosphorylation-dependent interaction of BACH1 and CtIP were shown in our lab to be dispensable for HDR and repair of ICL. In human cells, the phosphorylation-dependent interaction of these two BRCT phospho-proteins with BRCA1 can be ablated by a single amino acid substitution of the relevant serine for an alanine. Therefore, these two mutations were introduced into the corresponding Bach1 and Ctip genes in mouse ES cells to generate mice

homozygous for either mutant allele (Bach1$^{FHS994A/FHS994A}$, Ctip$^{S326A/S326A}$). MEFs homozygous for either mutation or both (Bach1$^{FHSA/FHSA}$, Ctip$^{SA/SA}$, Bach1$^{FHSA/FHSA}$Ctip$^{SA/SA}$) show limited sensitivity to genotoxic stress and are not deficient in HDR. However, the dependency of BRCA1 mediated DNA repair and tumorigenesis on its interaction with Abraxas remains unclear.

BRCA1 and the p53 binding protein, 53BP1, are involved in competing pathways to sites of DNA damage[7; 8]. Cells with a deficient Brca1 protein in a 53BP1$^{+/+}$ background are deficient in HDR. However, deletion of both 53BP1 alleles rescues the cell's ability to perform HDR[5]. Brca1$^{-/-}$ and Brca1$^{tr/tr}$ cells in a 53BP1$^{-/-}$ background are resistant to PARPi, showing similar chromosomal aberrations per metaphase as Brca1$^{+/+}$ 53BP1$^{-/-}$ control cells[5; 7]. However, Brca1 mutant cells in this background are still sensitive to ICL agents like MMC and cisplatin[5]. Since deletion of 53BP1 in Brca1 mutants seems to restore HDR, 53BP1 may compete with Brca1 in DNA damage response by NHEJ repair or other pathways of error-prone DNA repair and a second, unknown HDR pathway then responds to sites of DSBs when the Brca1 and 53BP1 pathways are unavailable[9]. Further, 53BP1 does not compete with Brca1 in repair of ICL damage since Brca1 mutants in a 53BP1$^{-/-}$ background are still sensitive to MMC and cisplatin.

The 53BP1$^{-/-}$ system we have established in mice is critical in elucidating BRCA1's specific role in the cell's DNA damage response. Since deletion of 53BP1 seems to restore HDR but not ICL repair related to the Brca1 pathway, we can use this system to our advantage when elucidating the role of BRCA1's phosphorylation-dependent interacting partners, Abraxas, BACH1, and CtIP, in DNA repair.

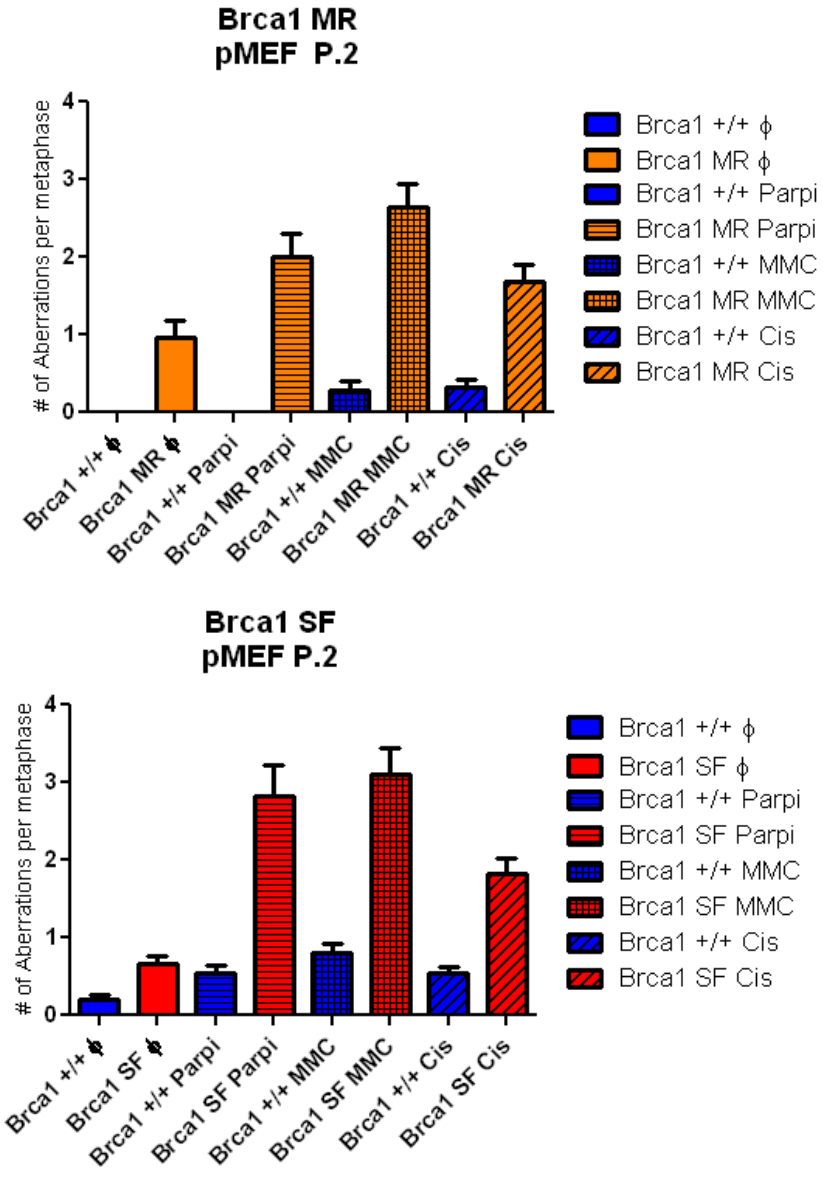


Figure 2: Brca1$^{MR/MR}$ and Brca1$^{SF/SF}$ MEFs are sensitive to genotoxic stress. A) Brca1$^{MR/MR}$ and B) Brca$^{SF/SF}$ MEFs accumulated spontaneous aberrations in untreated cultures and after treatment with Parp-i, MMC, and Cisplatin unlike Brca1$^{+/+}$ MEFs, which are resistant to DNA damaging agents.

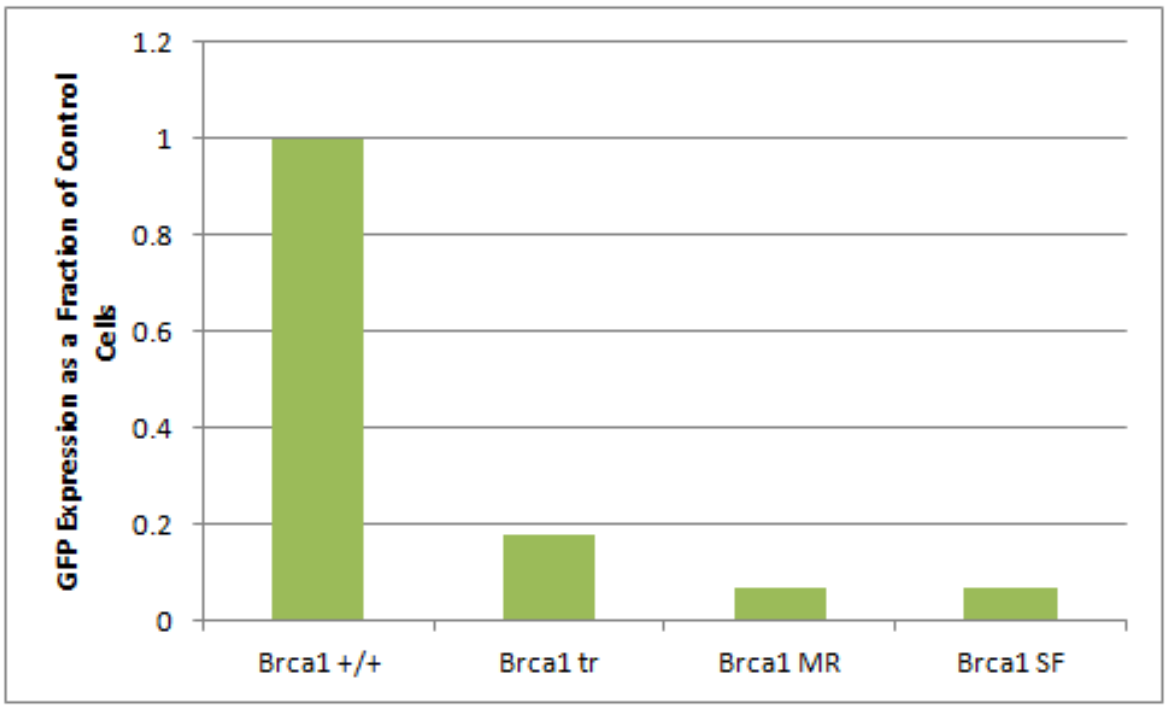


Figure 3. Brca1$^{tr/tr}$, Brca1$^{MR/MR}$, and Brca1$^{SF/SF}$ are deficient in HDR of DSBs. GFP expression as a fraction of Brca1$^{+/+}$ cells in Brca1$^{tr/tr}$ (0.18), Brca1$^{MR/MR}$ (0.07), and Brca1$^{SF/SF}$ (0.07) are significantly reduced compared to wildtype Brca1$^{+/+}$ cells.

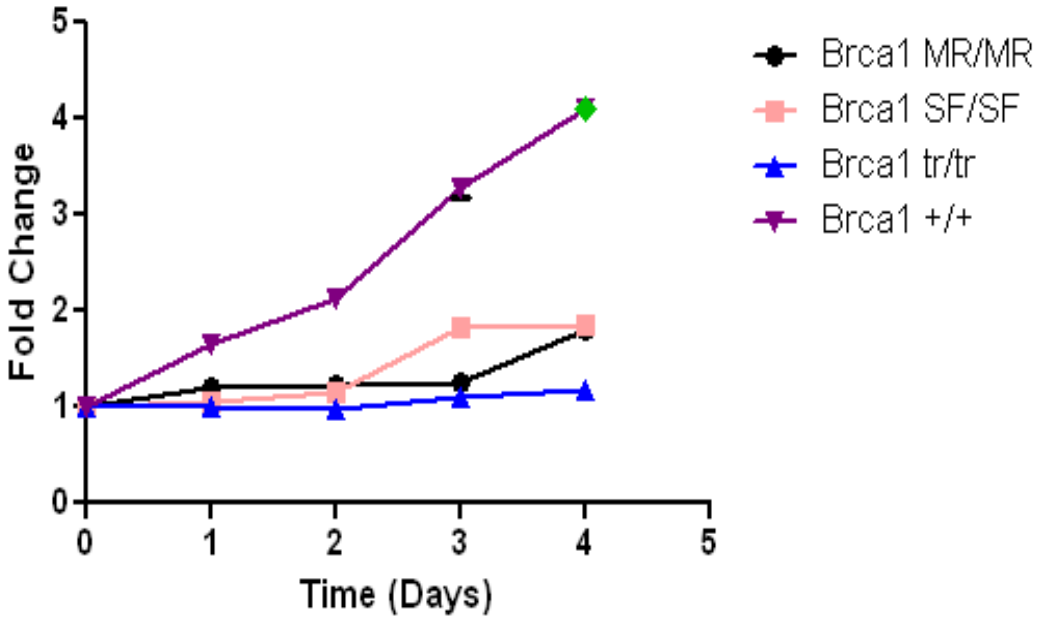


Figure 4. Brca1$^{tr/tr}$, Brca1$^{MR/MR}$, and Brca1$^{SF/SF}$ MEFs undergo proliferative arrest.
The various Brca1 mutant MEFs have decreased proliferation rates compared to Brca1$^{+/+}$ MEFs.

Methodology

In order to elucidate the mechanism by which BRCA1 facilitates DNA repair, I will investigate Brca1's phosphorylation-dependent interaction with Abraxas, Bach1, and Ctip in mice. Previously, our lab has generated mice with single or double point mutations in any of the three phospho-ligands. These point mutations result in a single amino acid substitution that ablates the phosphoserine interaction between Brca1 and the phospho-ligands resulting in mutant alleles Abx^{SA}, $Bach1^{FHSA}$, and $Ctip^{SA}$. Mice heterozygous or homozygous for one or two of the phospho-ligand mutant alleles were crossed to obtain homozygous single mutant $Abx^{SA/SA}$, double mutant $Bach1^{FHSA/FHSA}Ctip^{SA/SA}$ (BC), and triple mutant $Abx^{SA/SA}Bach1^{FHSA/FHSA}Ctip^{SA/SA}$ (ABC) MEFs. In addition, cells with the different genotypes were established in a $53BP1^{-/-}$ background. To elucidate the roles of each phospho-ligand in HDR of DSBs and ICL repair, mutant cells were treated with DNA damaging agents to observe chromosomal abnormalities in their karyotypes. To test the importance of these phospho-ligands specifically in HDR of DSBs, I have used the GFP fluorescence assay, which utilizes the DRGFP system. In addition, cell proliferation of the various genotypes will be measured by administering methylthiazole tetrazolium (MTT) to these cells and measuring relative absorbance values as a function of time. Cells were cultured at 37℃ in 3% O_2 and 5% CO_2.

Establishment of Mouse Embryonic Fibroblasts

Primary MEFs were derived from E13.5 embryos and cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 μg/mL penicillin/streptomycin, 2 mM L-glutamine, and 1.25 μg/mL Plasmocin at 37°C in 3% O_x and 5% CO_2. Yolk sacs and embryo heads were removed for genotyping by PCR and the viscera removed before establishment of MEF tissue onto 10 cm plates.

Karyotyping: Treatment with DNA Damaging Agents

Primary MEFs were seeded onto 10 cm plates ~24 hours prior to treatment with DNA damaging agents. The following cell densities were seeded: $4x10^6$ cells per plate for ABC 53BP1$^{+/+}$ cells and $2x10^6$ cells per plate for ABC 53BP1$^{-/-}$, Abx$^{SA/-}$, and control cells. Four plates per cell line were seeded to be untreated, treated with Parp-i (20 nM), MMC (60 nM), and cisplatin (5 nM). After 14 hours of incubation, cells were treated with 0.05 μg/mL of KaryoMAX Colcemid solution for 2 hours before harvesting. Cells were trypsinized, collected into 15 mL Falcon tubes and resuspended in 10 mL of 37°C 0.56% KCl solution for 30 minutes. After this incubation period, 1 mL of 3:1 methanol:glacial acetic acid was added to fix cells. Cell suspensions were centrifuged at 1000 rpm for 6 minutes and washed with the 3:1 MeOH: HOAc fixative. Metaphase spreads were prepared by dropping the cell suspension onto glass slides and staining with 1:10 KaryoMAX Geisma Stain in Gurr Buffer. Karyotypes of various cell-lines with different mutations and treated with Parp-i, MMC, and cisplatin were observed for chromosomal aberrations including chromatid breaks, chromosomal breaks, chromatid gaps, and exchanges. 25-50 metaphases were counted for each treatment.

Green Fluorescence Protein Assay

The green fluorescence protein assay utilizes the DR-GFP reporter gene (Figure 5) targeted into the mouse *Pim1* locus[4]. Repair of DSBs by HDR in dividing, somatic cells occurs primarily by a noncrossover gene conversion mechanism, which in the DR-GFP reporter restores a functional GFP gene[4]. The DR-GFP reporter gene contains two mutant GFP genes, the SceGFP gene located upstream of the iGFP gene[4]. The SceGFP gene contains a single point mutation that disrupts its coding potential of a functional GFP protein and is part of an 18 base pair recognition site for the I-SceI endonuclease[4]. The iGFP gene is truncated at both its 5' and 3' ends, thereby

lacking a promoter and cannot be transcribed[4]. I-SceI cleavage of SceGFP introduces a DSB, followed by HDR using the iGFP gene as a template. If HDR occurs, the coding sequence of a functional GFP protein is restored and cells fluoresce green, which can be detected by flow cytometry.

To analyze HDR in primary MEFs, the DR-GFP reporter was targeted into the Pim1 locus in mouse ES cells to derive chimeras heterozygous for DR-GFP. Heterozygous mice were intercrossed to obtain female and male mice homozygous for DR-GFP, which were mated with non-transgenic mice to derive $Pim1^{DRGFP/+}$ primary MEFs for HDR analysis.

Using this system, we were able to obtain $Abraxas^{SA/SA}Bach1^{FHSA/FHSA}Ctip^{SA/SA}53BP1^{+/+}$, $Abraxas^{SA/SA}Bach1^{FHSA/FHSA}Ctip^{SA/SA}53BP1^{-/-}$, $Abx^{SA/-}53BP1^{+/+}$, $Bach1^{FHSA/FHSA}Ctip^{SA/SA}53BP1^{+/+}$, $Bach1^{FHSA/FHSA}Ctip^{SA/SA}53BP1^{-/-}$ primary MEFs heterozygous for the DR-GFP gene. Passage 1 cells were collected and aliquoted into 4 electroporation cuvettes (1 cuvette with $2x10^6$ cells for IsceI–, 3 cuvettes with $3x10^6$ cells for IsceI+) to be electroporated (230 V, 950 mF) with either IsceI– (50 μg) or IsceI+(50 μg) expression vectors. Electroporated cells were seeded onto 10 cm plates and incubated for ~48 hours before analysis by flow cytometry. Approximately 48 hours after electroporation, cells were collected and resuspended in 1% FBS in PBS (500 μl) in preparation for fluorescence-activated cell sorting (FACS) analysis (100,000 events).

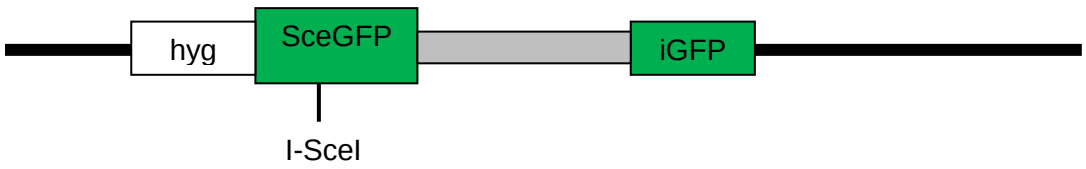


Figure 5. DR-GFP Reporter Gene

MTT Assay: Cell Proliferation

The MTT assay is a colorimetric assay used to assess cell proliferation. NAPDH-dependent oxioreductase enzymes in cells reduce MTT, a tetrazole, into formazan, which is purple in color[10]. Addition of solubilization solution dissolves the purple precipitate into solution to be analyzed by light absorbance. Mutant cell lines deficient in cell proliferation will have smaller absorbance values relative to control cell lines.

Passage 1 cells were seeded onto 5 (Day 0 to Day 4) 96-well plates (1000 cells/well, 2000 cells/well) and cultured at 37°C in 3% O_x and 5% CO_2. 100 µl of 0.5 mg/mL MTT in DMEM was aliquoted into each well and incubated under the above conditions for 3 hours, after which 100 µl of solubilization solution (10% SDS, 0.01 M HCl) was added into each well. ~24 hours after MTT addition, plates were analyzed by obtaining absorbance values at 495 nm. Day 0, 1, 2, 3, and 4 plates were treated with MTT 24, 48, 72, 96, and 120 hours after seeding, respectively. Absorbance values were averaged and normalized to their corresponding Day 0 values.

Results

Sensitivity to Genotoxic Stress

Brca1$^{SF/SF}$ cells, which contain a missense mutation (S1598F) that ablates the BRCT phospho-recognition activity of Brca1, are hypersensitive to ICL agents MMC and cisplatin and to ssDNA repair inhibitor Parp-i. However, Bach1$^{FHSA/FHSA}$, Ctip$^{SA/SA}$, and Bach1$^{FHSA/FHSA}$Ctip$^{SA/SA}$ cells, which contain a missense mutation that ablates the phospho-recognition activity of Bach1, Ctip, or both, respectively, show limited sensitivity to MMC and Parp-i. This suggests that while cellular resistance to MMC, cisplatin, and Parp-i is dependent on BRCA1's interaction with one or more of its BRCT phospho-ligands, the phosphorylation-dependent interaction of BACH1 and CtIP with BRCA1 are dispensable.

Cells expressing the Abraxas-SA mutant show limited sensitivity to genotoxic stress.

To determine whether the BRCA1-Abraxas interaction is required for resistance to MMC, cisplatin, and Parp-i, Abx$^{SA/–}$ and Abx$^{SA/+}$ MEFs were treated with the above DNA damaging agents. As shown in (Fig 6) the number of aberrations per metaphase for Abx$^{SA/–}$ cells are similar in frequency to those of Abx$^{SA/+}$ control cells for all drug treatments. Thus, although resistance to MMC, cisplatin, and Parp-i are dependent on intact BRCA1 BRCT repeats, ablation of the phospho-dependent interaction of Abraxas with Brca1, like Bach1 and CtiP, does not result in sensitivity to the DNA-damaging agents.

Abx$^{SA/SA}$Bach1$^{FHSA/FHSA}$Ctip$^{SA/SA}$ triple mutant cells are hypersensitive to genotoxic stress.

BRCA1's phosphorylation-dependent interaction solely with either Abraxas, Bach1, or CtIP is dispensable for resistance to MMC, cisplatin, and Parp-i, however, BRCA1's ability to interact with its cognate BRCT phospho-ligands is required for cellular resistance to these three DNA damaging agents. To determine whether Abraxas, Bach1, and CtIP together are required

for resistance to MMC, cisplatin, and Parp-i, triple mutant MEFs ($Abx^{SA/SA}Bach1^{FHSA/FHSA}Ctip^{SA/SA}$) were treated with all three DNA damaging agents. Triple mutant ABC cells showed significantly greater frequencies of aberrations per metaphase compared to control double mutant BC and $Brca1^{+/+}$ cells, including spontaneous aberrations even in the absence of drug treatment (Fig 7, 9a,b). These frequencies were similar to those observed in $Brca1^{SF/SF}$ cells (Fig. 9a,b). Thus, while BRCA1's independent interaction with Abraxas, Bach1, and CtIP is dispensable for resistance to these three DNA damaging agents, concomitant ablation of all three phospho-dependent interactions with BRCA1 results in drug sensitivity.

$Abx^{SA/SA}Bach1^{FHSA/FHSA}Ctip^{SA/SA}$ triple mutant cells in a $53BP1^{-/-}$ background are resistant to DNA damaging agents.

$Brca1^{tr/tr}$, $Brca1^{M1717R/M1717R}$ and $Brca1^{S1598F/S1598F}$ MEF cells are sensitive to ICL agents MMC and cisplatin as well as the ssDNA repair inhibitor Parp-i[5].

However, in a $53BP1^{-/-}$ background these cells become resistant to the ssDNA repair inhibitor Parp-I, but remain sensitive to ICL agents MMC and cisplatin. This suggests that while in the absence of 53BP1 BRCA1's phosphorylation-dependent interaction with its cognate phospho-ligands is dispensable for resistance to Parp-i, it is still required for resistance to ICL agents. To determine whether sensitivity of triple mutant ABC cells to these DNA damaging agents can be rescued in a $53BP1^{-/-}$ background, ABC $53BP1^{-/-}$ mutant MEFs were in parallel with BC $53BP1^{-/-}$ control cells. The frequency of aberrations per metaphase for ABC $53BP1^{-/-}$ cells are similar to those of BC $53BP1^{-/-}$ and $Brca1^{+/+}$ control cells, unlike ABC $53BP1^{+/+}$ and $Brca1^{SF/SF}$ cells (Fig 8, 9a,b). Thus, the sensitivity of ABC MEFs to interstrand crosslinking agents as well as ssDNA repair inhibitor Parp-I can be rescued in a 53BP1-deficient background.

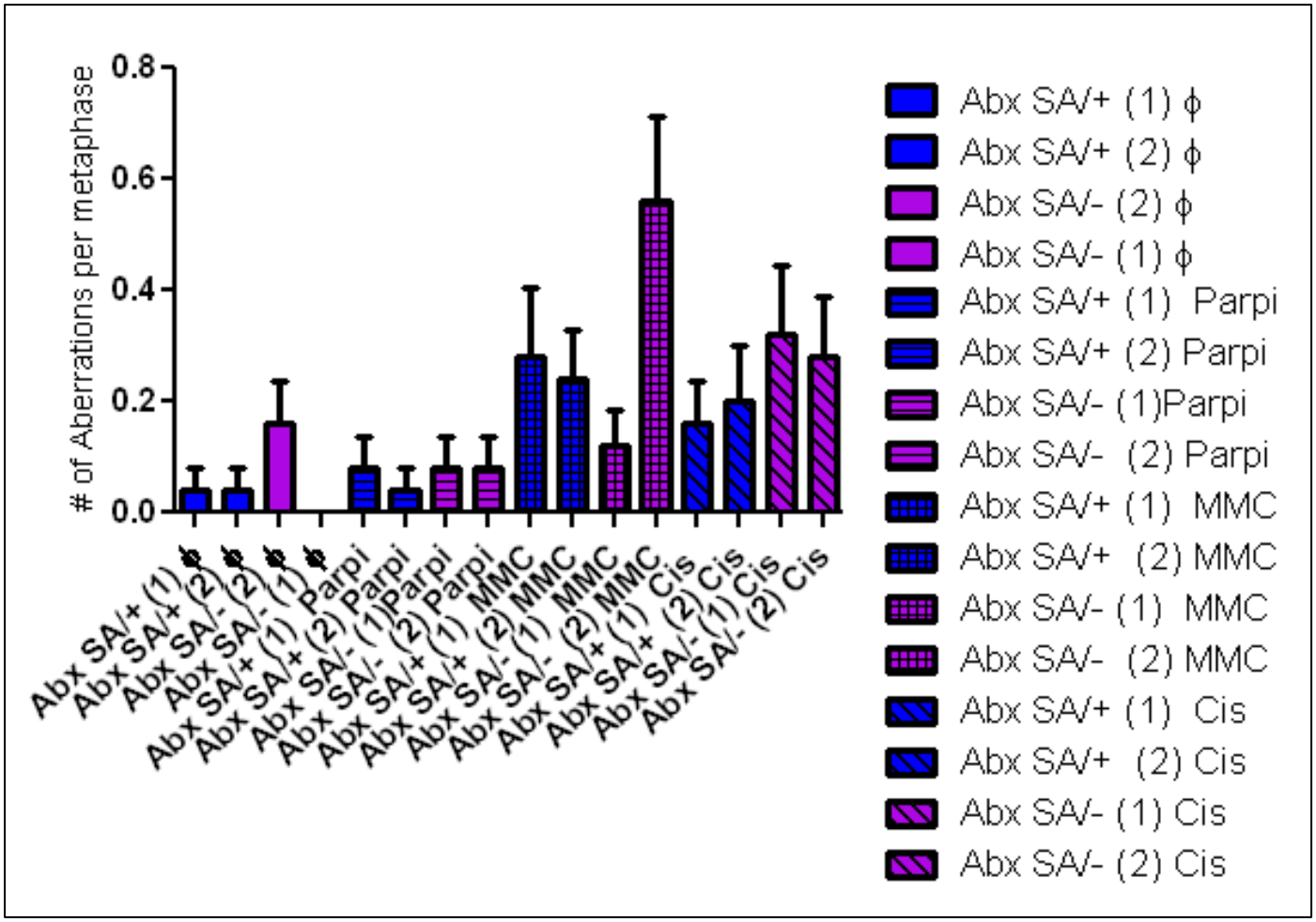


Figure 6. Abraxas$^{SA/-}$ MEFs show limited sensitivity to genotoxic stress.

Abraxas$^{SA/-}$ cells exhibited frequency of aberrations per metaphase similar to those of Abraxas$^{SA/+}$ control cells in untreated cells and in cells treated with Parp-i, MMC, and cisplatin. The number of aberrations per metaphase for each cell line are as follows: Abraxas$^{SA/-}$ (0-0.16 untreated, 0.08 Parp-i, 0.12-0.56 MMC, 0.28-0.32 Cis), Abraxas$^{SA/+}$ (0.04 untreated, 0.04-0.08 Parp-i, 0.24-0.28 MMC, 0.16-0.20 Cis).

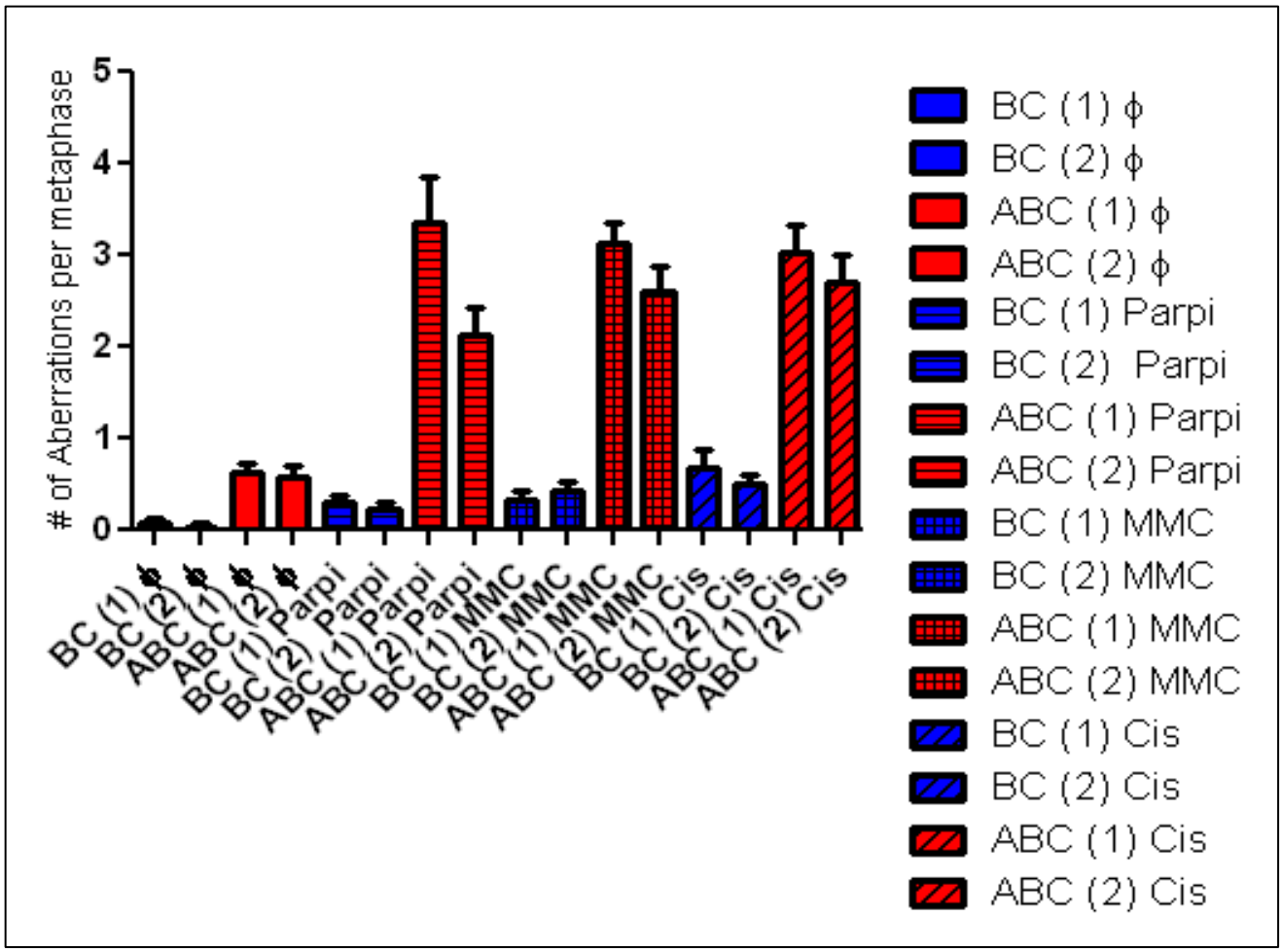


Figure 7. Triple mutant ABC MEFs are hypersensitive to genotoxic stress.
ABC MEFs accumulated spontaneous aberrations in untreated cultures and after treatment with Parp-i, MMC, and cisplatin unlike BC control MEFs, which are resistant to DNA damaging agents. The number of aberrations per metaphase for each cell line are as follows: ABC (0.58-0.64 untreated, 2.14-3.34 Parp-i, 2.60-3.12 MMC, 2.70-3.04 Cis), BC (0.04-0.08 untreated, 0.22-0.30 Parp-i, 0.34-0.42 MMC, 0.50-0.68 Cis). 25 metaphases were counted per treatment.

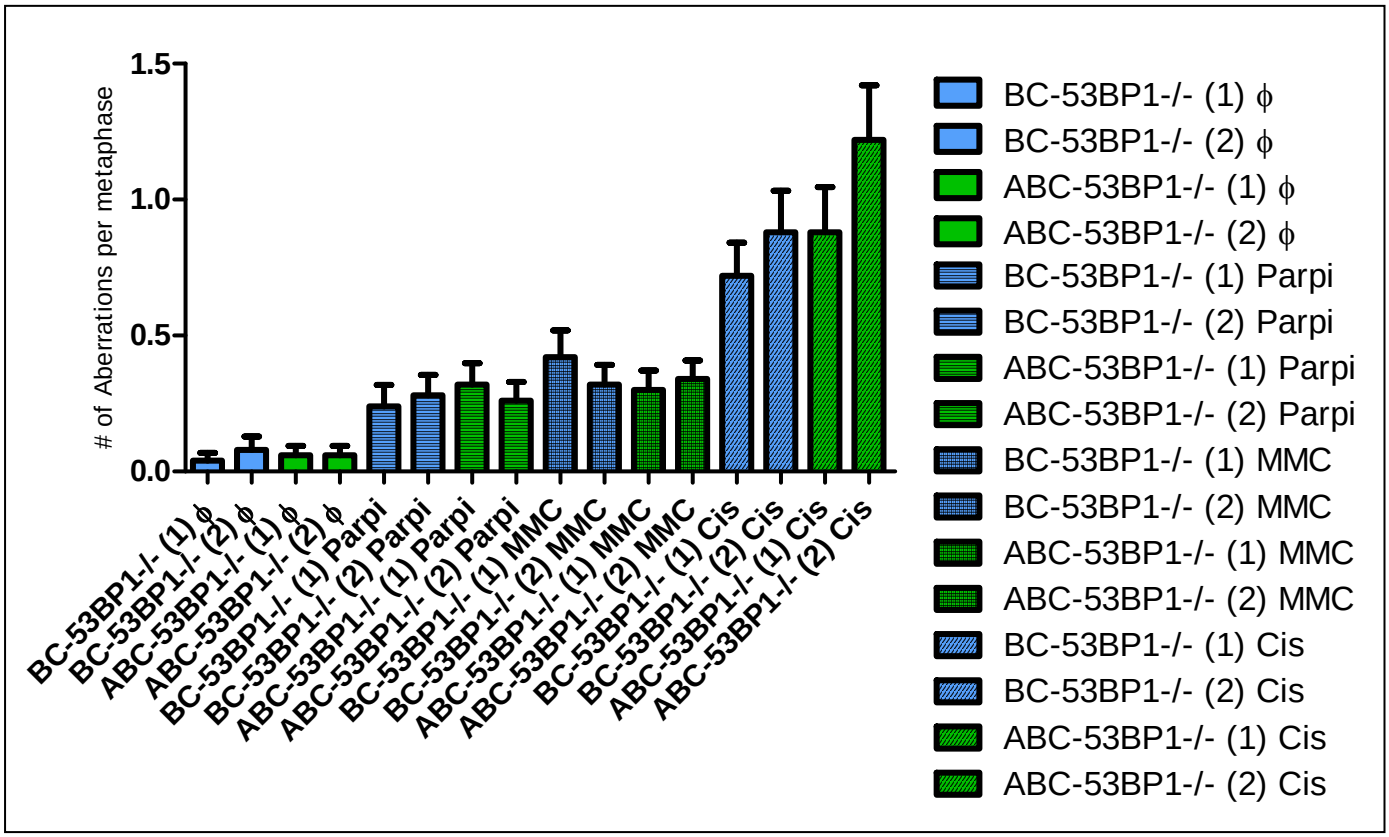
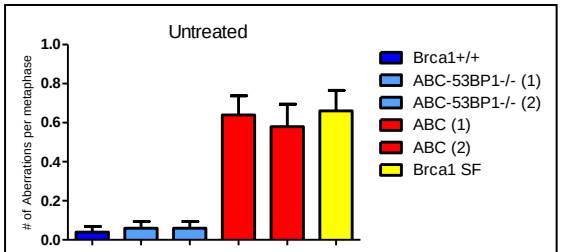
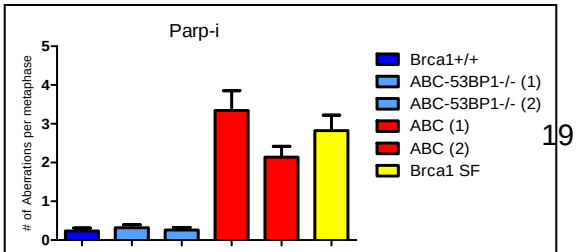
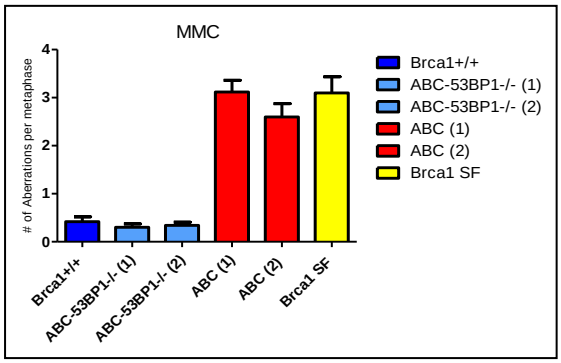


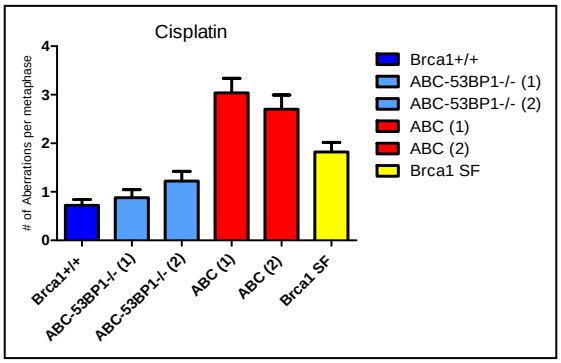
Figure 8. ABC 53BP1$^{-/-}$ MEFs show limited sensitivity to genotoxic stress.
ABC 53BP1$^{-/-}$ cells exhibited frequency of aberrations per metaphase similar to those of BC 53BP$^{-/-}$ control cells for untreated cultures and Parp-i, MMC, and cisplatin treated cultures. The number of aberrations per metaphase for each cell line are as follows: ABC 53BP1$^{-/-}$ (0.06 untreated, 0.26-0.32 Parp-i, 0.30-0.34 MMC, 0.88-1.22 Cis), BC 53BP$^{-/-}$ (0.04-0.08 untreated, 0.24-0.28 Parp-i, 0.32-0.42 MMC, 0.72-0.88 Cis). 50 metaphases were counted per treatment.

A.









B. Number of Aberrations per Metaphase

	$Brca1^{+/+}$ (1)	ABC $53BP1^{-/-}$ (1)	ABC $53BP1^{-/-}$ (2)	ABC (1)	ABC (2)	Brca1 SF
ø	0.04	0.06	0.06	0.64	0.58	0.66
Parp-i	0.24	0.32	0.26	3.34	2.14	2.82
MMC	0.42	0.30	0.34	3.12	2.60	3.10
Cis	0.72	0.88	1.22	3.04	2.70	1.82

Figure 9. Frequency of aberrations per metaphase for ABC $53BP1^{-/-}$, ABC $53BP1^{+/+}$, $Brca1^{SF/SF}$, and $Brca1^{+/+}$ MEFs untreated and after treatment with Parp-i, MMC, and Cis. A) ABC $53BP1^{+/+}$ cells are hypersensitive to genotoxic stress and exhibit frequency of aberrations per metaphase similar to $Brca1^{SF/SF}$ cells, unlike ABC $53BP1^{-/-}$ cellS, which exhibit aberration frequencies similar to control $Brca1^{+/+}$ cells. B) Frequency of chromosomal damage are listed as number of aberrations per metaphase. 50 metaphases were counted per treatment.

HDR pathway of DSB repair

$Brca1^{SF/SF}$ cells were shown to be deficient in HDR and showed significant reductions in expression of GFP positive cells compared to $Brca1^{+/+}$ control cells using the DR-GFP HDR assay. Double mutant BC and $Brca1^{+/+}$ cells showed similar percentages of GFP positive cells. These results indicate that BRCA1's BRCT phosphorylation-dependent interaction with one or more of its cognate phospho-ligands is essential for HDR of DSBs, however, its interactions with Bach1 and CtIP alone are dispensable.

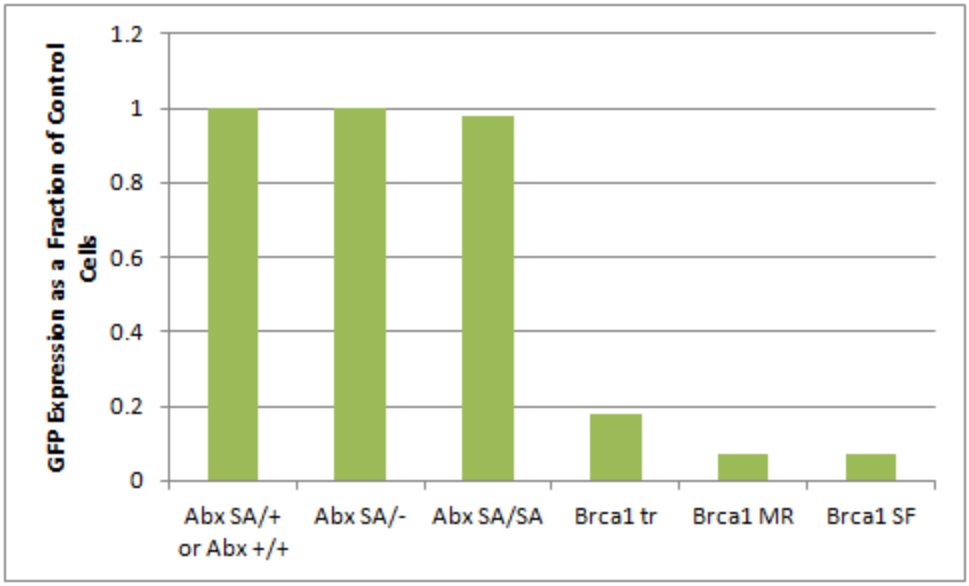
To measure HDR in cells, MEFs of the various genotypes were subjected to the DRGFP HDR assay and using flow cytometry to measure the percentage of GFP positive cells, HDR could be quantified. Due to the variability of absolute percentages of control cells from experiment to experiment in this assay, mutant cell GFP percentages were normalized to those of the control cell lines for each experiment. The data is presented by measuring GFP expression of mutant cell lines as a percentage of their respective controls (controls are set to a value of 1). In the absence of I-SceI, GFP-positive cells were rarely detected (~0%) in all control and mutant cell lines, indicating that spontaneous recombination is rare.

The Brca1-Abraxas interaction is not essential for HDR of DSBs.

To determine whether the BRCA1-Abraxas interaction is required for HDR of DSBs, we established $Abx^{SA/-}$,$Abx^{SA/SA}$, and control $Abx^{SA/+}$ and $Abx^{+/+}$ MEFs that were also heterozygous for DRGFP and performed the DRGFP HDR assay on these cells. As shown in Figure 10a, $Abx^{SA/-}$ and $Abx^{SA/SA}$ cells exhibited similar levels of GFP positive cells (0.98-1.00) as $Abx^{SA/+}$ and $Abx^{+/+}$ control cells, unlike $Brca1^{tr/tr}$, $Brca1^{MR/MR}$, and $Brca1^{SF/SF}$ cells (0.18, 0.07, 0.07). These results suggest that HDR of DSBs is not dependent on BRCA1's interaction with Abraxas.

Abx$^{SA/SA}$Bach1$^{FHSA/FHSA}$Ctip$^{SA/SA}$ triple mutant cells are deficient in HDR of DSBs.

While BRCA1's phosphorylation-dependent interaction exclusively with either Abraxas, Bach1, and CtIP are dispensable for HDR of DSBs, it remained unclear as to whether combinations of these mutually exclusive interactions was necessary for HDR. Therefore, to determine whether Abraxas, Bach1, and CtIP together were necessary for BRCA1 mediated HDR of DSBs, triple mutant ABC and double mutant BC MEFs heterozygous for DRGFP were subjected to the DRGFP HDR assay. As shown in Figure 10b, ABC triple mutants cells exhibited decreased levels of GFP expression (0.11) relative to BC double mutant control cell lines and similar levels to those of Brca1 BRCT mutant MEFs. These results indicate that while BRCA1's BRCT phosphorylation-dependent interaction exclusively with either Abraxas, Bach1, or CtIP is dispensable for HDR of DSBs, BRCA1's interaction with all three phospho-ligands is required for functional HDR of DSBs.



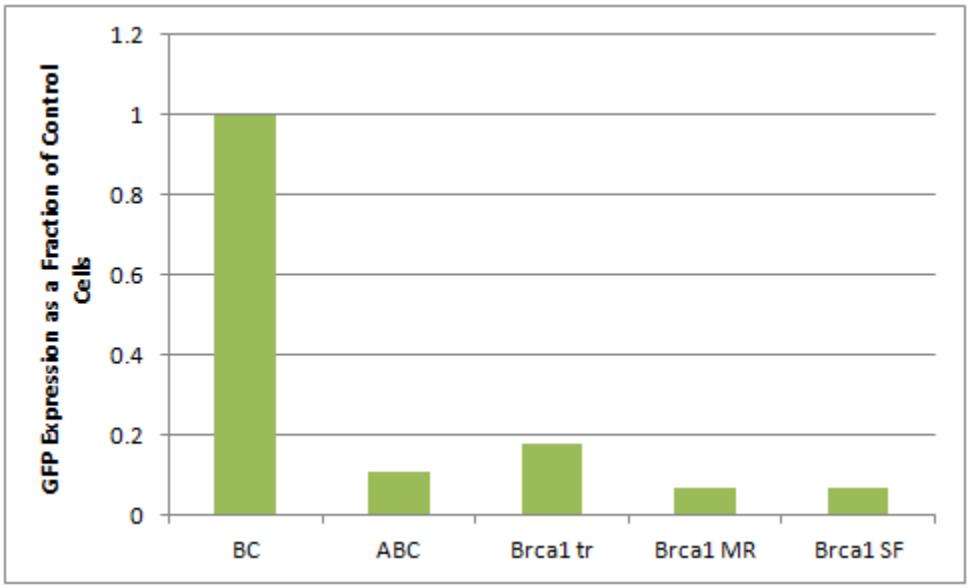


Figure 10. HDR of DSBs in Abraxas$^{SA/SA}$, Abraxas$^{SA/-}$, and ABC 53BP1$^{+/+}$ MEFs.
A) Abraxas$^{SA/SA}$ and Abraxas$^{SA/-}$ cells are not deficient in HDR of DSBs and exhibit similar levels of GFP expression (0.98, 1.00) to that of Abraxas$^{+/+}$ and Abraxas$^{SA/+}$ control cells . B) ABC 53BP1$^{+/+}$ cells are deficient in HDR of DSBs and exhibit reduced levels of GFP expression (0.11) compared to that of BC 53BP1$^{+/+}$ control cells.

Cell Proliferation

Abraxas$^{SA/SA}$ and ABC 53BP1$^{-/-}$ cells proliferate at normal rates while ABC 53BP1$^{+/+}$ cells undergo proliferative arrest.

Brca1$^{SF/SF}$ MEFs undergo proliferative arrest relative to control Brca1$^{+/+}$ cell lines, indicating that BRCA1's phosphorylation-dependent interaction with one or more of its BRCT phospho-ligands is necessary for normal proliferation. However, double mutant BC cells proliferate at rates similar to wildtype Brca1$^{+/+}$ cells, indicating that BRCA1's exclusive interaction with either Bach1 or CtIP are dispensable for normal proliferation.

To determine whether normal cellular proliferation is dependent on the BRCA1-Abraxas interaction, passage 2 Abx$^{SA/SA}$ mutant MEFs were evaluated in the MTT assay in parallel with Abx$^{+/+}$ control MEFs. As shown in (Fig 11a), the proliferation curves of Abx$^{SA/SA}$ cells overlap with those of control Abx$^{+/+}$ cells. This suggests that BRCA1's exclusive interaction with Abraxas, much like with that of Bach1 and CtIP, is dispensable for normal proliferation.

Based, on this results, it seems that BRCA1's exclusive interaction with either of the three phospho-ligands is not essential for normal cellular proliferation. However, it remained unclear as to whether BRCA1's phosphorylation-dependent interaction with more than one of the three phospho-ligands was important for normal cellular proliferation. To evaluate this, triple mutant ABC MEFs were evaluated in the MTT assay in parallel with double mutant BC MEFs. As shown in Figure 11b, the proliferation curve for the ABC cells has a nearly horizontal slope while the proliferation curve for the BC cells increases steadily, implicating that BRCA1's interaction with all three phospho-ligands is necessary for normal cellular proliferation.

Because Brca1 mutants in a 53BP1$^{-/-}$ background exhibit normal proliferation rates, we set out to determine whether a 53BP1$^{-/-}$ background would similarly rescue proliferation deficiencies seen in ABC mutant cells. As shown in Figure 11c, ABC 53BP1$^{-/-}$ MEFs evaluated

in the MTT essay show overlapping proliferation curves with those of BC 53BP1$^{-/-}$ control MEFs.

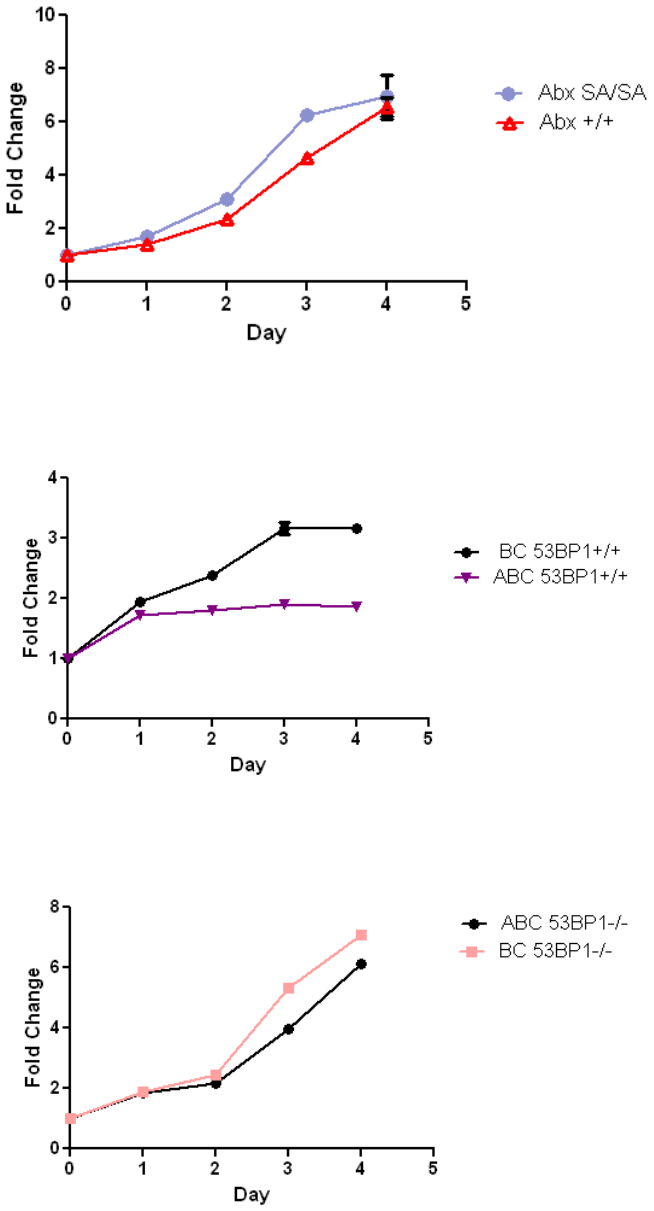


Figure 11. Cellular Proliferation Curves for Abx$^{SA/SA}$, ABC 53BP1$^{+/+}$, and ABC 53BP1$^{-/-}$ MEFs

A) Abx$^{SA/SA}$ cells proliferate normally, similar to control Abx$^{+/+}$ cells unlike B) ABC mutant cells, which undergo proliferative arrest relative to control BC cells. C) ABC mutant cells in a 53BP1$^{-/-}$ background proliferate normally, similar to control BC 53BP1$^{-/-}$ cells.

Discussion

Prior evidence indicated that the BRCT domains of BRCA1 are essential for HDR of DSBs. Specifically, the phosphorylation-dependent interaction of the BRCT domain with its cognate phospho-ligands is necessary. Previous studies and our own lab have showed that Brca1 BRCT mutants ($Brca1^{tr/tr}$, $Brca1^{M1717R/M1717R}$, and $Brca1^{S1598F/S1598F}$) are deficient in HDR repair of DSBs compared to control $Brca1^{+/+}$ cells. Additionally, Brca1's interaction with Bach1 and Ctip were dispensable for HDR. Our results show that $Abraxas^{SA/SA}$ and $Abraxas^{SA/-}$ showed efficient HDR similar to that of $Abraxas^{SA/+}$ and $Abraxas^{+/+}$ control cells. Interestingly, ablating the ability of all three BRCT phospho-ligands to bind to Brca1 resulted in HDR deficiency. This supports the hypothesis that BRCA1 forms three mutually exclusive protein complexes with Abraxas, Bach1, and CtIP. Ablating the interaction of just one phospho-ligand does not result in HDR deficiency, but ablating all three phospho-ligands' ability to bind Brca1 results in HDR deficiency. This suggests that the complexes BRCA1 forms through the phospho-dependent interactions with Abraxas, Bach1, and CtIP are functionally redundant for HDR and loss of one or two interactions can be compensated for.

The HDR data is supported by the cell proliferation data obtained from the MTT assay. Brca1 BRCT mutant cells senesce early on compared to $Brca1^{+/+}$ cells, indicating that the phosphorylation dependent activity of the BRCT repeats in BRCA1 are important for normal cell proliferation. $Abraxas^{SA/SA}$ cells, like $Brca1^{+/+}$ and double mutant BC cells, proliferate normally, indicating that the BRCA1-Abraxas interaction is dispensable for normal cell proliferation. However, ablating all three phospho-ligands' abilities to bind Brca1 results in proliferative arrest, similar to those of Brca1 BRCT mutant cells. The positive correlation between cellular

proliferation and HDR capability in cells suggests that maintenance of genome stability is key in normal cellular proliferation.

BRCA1 in human cells is implicated in repair of DNA lesions, including DSBs and ICL damage. Brca1$^{SF/SF}$ cells are hypersensitive to genotoxic stress, indicating that resistance to ICL agents and Parp inhibitors is dependent on BRCA1's phosphorylation-dependent interaction with one or more of its phospho-ligands. However, cells expressing Bach1^{FHSA} or Ctip^{SA} mutant alleles showed no discernible sensitivity to genotoxic stress, indicating that BRCA1's interaction with BACH1 or CtIP is dispensable for resistance to DNA damaging agents. We found that Abraxas$^{SA/-}$ cells were resistant to ssDNA repair inhibitor and ICL agents, indicating that the interaction between BRCA1 and Abraxas exclusively also is not required for HDR of DSBs and ICL damage repair. However, in triple mutant ABC cells, treatment with Parp-i, MMC, and cisplatin result in significant accumulation of chromosomal aberrations compared to control cells. This supports the HDR data and suggests that BRCA1's phosphorylation-dependent interaction with Abraxas, Bach1, and CtIP plays key roles in repairing DNA lesions, but just one pathway is sufficient.

While the mentioned results give us great insight into the BRCA1-mediated DNA repair pathways, specifically HDR, it was still unclear as to how BRCA1 mediates HDR and ICL repair. We know Brca1 BRCT mutants are hypersensitive to both ssDNA repair inhibitor, Parp-i, and ICL agents, MMC and cisplatin. However, Brca1$^{tr/tr}$, Brca1$^{M1717R/M1717R}$, and Brca1$^{S1598F/S1598F}$ cells in a 53BP1$^{-/-}$ background are resistant to Parp-i, but still sensitive to MMC and cisplatin[5]. Deletion of 53BP1 in Brca1 BRCT mutants evidently rescues HDR, but not repair of ICLs.

We used this system to our advantage to determine whether BRCA1 mediates both types of repair through its interaction with Abraxas, Bach1, and CtIP. Interestingly, in contrast to Brca1 BRCT mutant/53BP1$^{-/-}$ cells, triple mutant ABC cells in a 53BP1$^{-/-}$ background are not only resistant to Parp-i, but also to the interstand crosslinkers MMC and cisplatin. This suggests that HDR is restored in triple mutant cells in the absence of 53BP1, indicating that a second HDR pathway repairs DSBs in the absence of BRCA1 binding to Abraxas, BACH1, or CtIP. However, unlike Brca1 BRCT mutants that are null for 53BP1, ABC 53BP1$^{-/-}$ cells are also resistant to ICL agents. As mentioned before, ABC 53BP1$^{+/+}$ cells are hypersensitive to both Parp-i and ICL agents, indicating that BRCA1's interaction with at least one of the three phospho-ligands is required for HDR and repair of ICLs. This suggests that BRCA1 must mediate repair of ICL damage through its interaction with either of the three phospho-ligands at a downstream point in this pathway, but mediates ICL repair at an upstream point independent of its interaction with Abraxas, BACH1, and CtIP. A possible mechanism for BRCA1 mediated repair of ICL damage is that at some upstream point in this pathway, ICL damage is converted into a DSB, thus requiring HDR at some downstream point. Since ABC 53BP1$^{+/+}$ cells are deficient in HDR and ICL repair, it is possible that BRCA1's interaction with either Abraxas, BACH1, or CtIP is required in HDR at some downstream point in ICL repair. Because Brca1 BRCT mutants in a 53BP1$^{-/-}$ background are sensitive to ICL agents while ABC 53BP1$^{-/-}$ mutants are not, repair of ICL damage at some upstream point must be BRCA1 mediated but independent of its interaction with either of the three phospho-ligands and a second HDR pathway is able to perform HDR at some downstream point in ICL repair (Figure 12).

Sufficient evidence exists that suggests BRCA1's interaction with just one of the three phospho-ligands is adequate for HDR and ICL repair, since single mutant Abraxas^{SA}, Bach1^{SA},

and Ctip^{SA} cells are not HDR deficient and are resistant to DNA damaging agents while triple mutant ABC cells are HDR deficient and hypersensitive to DNA damaging agents. Furthermore, it has been shown that when the phosphorylation-dependent interaction specifically between Bach1 and Ctip with Brca1 are disrupted simultaneously, cells are still able to perform HDR and are resistant to DNA damaging agents. However, to strengthen this model, double mutant Abraxas^{SA}Ctip^{SA} and Abraxas^{SA}Bach1^{SA} cells need to be tested for HDR, cell proliferation, and sensitivity to DNA damaging agents. Additionally, our results suggest that ABC 53BP1$^{-/-}$ cells are resistant to Parp-i, thus can perform HDR like wild type cells. To confirm this theory in the future, ABC 53BP1$^{-/-}$ cells need to be evaluated in the DRGFP HDR assay.

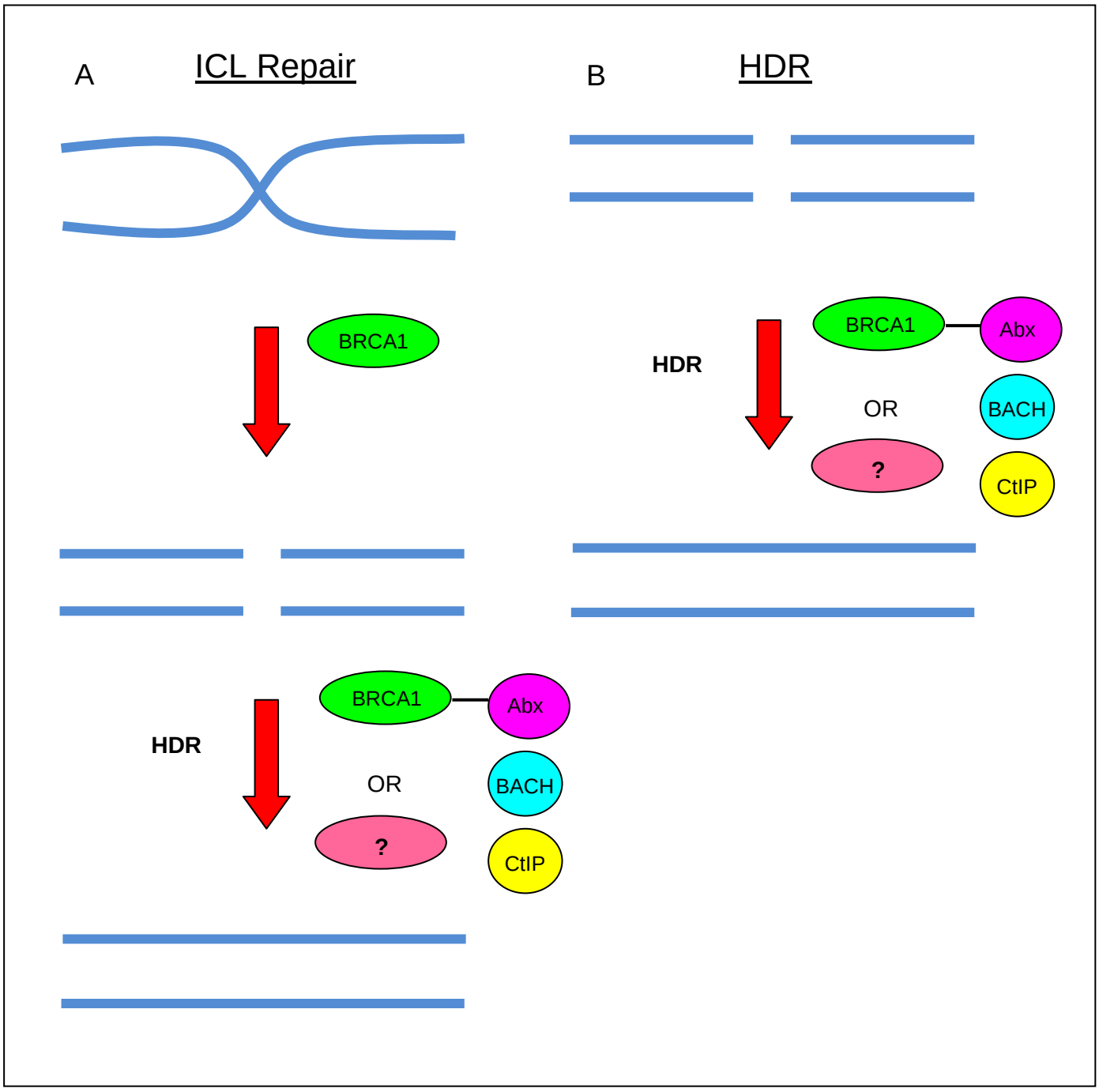


Figure 12. BRCA1 mediated ICL repair and HDR of DSBs.
A) Repair of ICL damage at an upstream point converts ICLs to DSBs and HDR follows. BRCA1's interaction with either Abraxas, BACH1, or CtIP facilitates repair of ICLs via HDR at this downstream point in a 53BP1$^{+/+}$ background, but second HDR pathway follows in absence of 53BP1. B) HDR of DSBs is mediated by BRCA1 and its cognate phospho-ligands in a 53BP1$^{+/+}$ background, but not in a 53BP1$^{-/-}$ background.

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